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THE NATURE OF THE FORCES BETWEEN ANTIGEN AND ANTIBODY AND OF THE PRECIPITATION REACTION

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In one of his lectures on immunochemistry at the University of California in the summer of 1904 Svante Arrhenius said (1) that Ehrlich and other investigators, because of incomplete knowledge of the phenomenon of chemical equilibrium, had been led to invent artificial hypotheses in order to explain their observations in the field of immunology. Since that time, and especially during the last few years, workers in this field have made greater and greater use of the concepts and methods of physical chemistry, and in consequence many previously puzzling observations have been reasonably interpreted.

Another branch of chemistry which is of importance to immunology is modern structural chemistry, which deals with the detailed structure of molecules and with the nature of interatomic and intermolecular interactions (2). Our present knowledge of this subject, in large part won during the past dozen years, is now so firmly founded and so extensive that it can be confidently used as the basis for a more penetrating interpretation of immunological observations than would be provided by the observations alone.

In this paper we present, after a brief historical introduction, a discussion of the nature of the specific forces between antigen and antibody and of the precipitation reaction from the point of view of modern chemistry. Only the simpler aspects of the phenomena are discussed; such complicating factors as the rôles of complement, lipids, etc., in the reactions are disregarded in our discussion.

The history of the precipitation reaction began in 1897, when Rudolf Kraus (3) reported the results of his work with anticholera and antityphoid sera. His observations were soon verified and extended by Nicolle, Tchistovich, Bordet, Myers and other workers, who prepared precipitating antisera against a great number of antigens of varied nature. We shall not review this early work here, nor the later studies of the methods of preparing antisera and carrying out the precipitation reaction, since these topics and others dealing with special phases of the reaction have been very well covered in earlier reviews (4, 5, 6).

Two most important advances in the attack on the problem of the nature of immunological reactions were the discovery that the specific precipitate contains both antigen and antibody (7) and the discovery that antibodies, which give antisera their characteristic properties, are proteins. The verification of these facts was provided by the work of many investigators over a score of years. This work, which is summarized in Marrack's monograph (6, chap. II), culminated in the preparation of purified antibody by Felton and Bailey (8), Heidelberger and collaborators (9), and others, and the determination of its properties, including amino-acid composition and molecular weight, which show that it is very closely related to normal serum globulin (6, chap. II).

The work of Landsteiner (10) and other investigators on artificial conjugated antigens provided a great body of qualitative information on the specificity of antibodies, which, together with the experimental results for natural antigens, led to the independent proposal by Breinl and Haurowitz (11), Alexander (12), and Mudd (13) in 1930–32 of the theory of structural complementariness of antigen and specific antibody. The framework theory of precipitation was then developed by Marrack (6) and Heidelberger (14). These and other theories are discussed in some detail in the following sections of this paper.

A new period in the study of the precipitation reaction was initiated by the careful quantitative studies of Heidelberger and his collaborators (15) who determined the amounts of antibody and antigen in precipitates, and the similar work of Haurowitz (16) and others. Very recently, in order to test certain aspects of his detailed theory of the structure of antibodies (17), Pauling and his collaborators have carried out many quantitative experiments on the precipitation of antisera by polyhaptenic simple substances (18, 19, 20), a phenomenon first observed by Landsteiner and Van der Scheer (21).

THE NATURE OF THE SPECIFIC FORCES BETWEEN ANTIGEN MOLECULES AND ANTIBODY MOLECULES. The detailed information which has been gathered in recent years regarding the nature of the chemical bonds which hold atoms together into stable molecules has been summarized in monographs (2, 22). Instead of interacting strongly with one another, with interaction energy of 20 kilocalories per mole or more, to produce a chemical bond, two atoms may interact more weakly. The nature of these weak interactions is now well understood, and a brief discussion of it is given in the following paragraphs. The properties of antigen-antibody systems, especially the reversibility of complex formation, are such as to indicate that the antigen-antibody attraction is due to these weaker interactions and not to the formation of ordinary chemical bonds.

The weak interactions between two molecules may be classified as electronic van der Waals attraction, Coulomb attraction, attraction of electric dipoles or multipoles, hydrogen-bond formation, etc. The forces increase rapidly in magnitude as the molecules approach one another more and more closely, and the attraction between the molecules reaches its maximum when the molecules are as close together as they can come. The molecular property which determines the distance of closest approach of two molecules is the electronic spatial extension of the atoms in the molecules. It is possible to assign to each atom a *van der Waals radius*, which describes its effective size with respect to intermolecular interactions. These radii vary in value from 1.2 Å for hydrogen through 1.4–1.6 Å for light atoms (fluorine, oxygen, nitrogen, carbon) to 1.8–2.2 Å for heavy atoms (chlorine, sulfur, bromine, iodine, etc.). The shape of a molecule can be predicted by locating the atoms within the molecule with use of bond distances and bond angles and then circumscribing about each atom a spherical surface corresponding to its van der Waals radius. This shape determines the ways in which the molecule can be packed together with other molecules (2, sec. 24).

The most general force of intermolecular attraction, which operates between

every pair of molecules, is *electronic van der Waals attraction.* This type of electronic interaction between molecules was first recognized by London (23). A molecule (of methane, for example) which has no permanent average electric dipole moment may have an instantaneous electric dipole moment, as the center of charge of the electrons, in their rapid motion in the molecule, swings to one side or the other of the center of charge of the nuclei. This instantaneous dipole moment produces an instantaneous electric field, by which any other molecule in the neighborhood would be polarized; the electrons of the second molecule would move relative to its nuclei in such a way as to give rise to a force of attraction toward the first molecule.

This electronic van der Waals attraction operates between every atom in a molecule and every atom in other molecules in the near neighborhood. The force increases very rapidly with decreasing interatomic distance, being inversely proportional to the seventh power of the interatomic distance. Hence the electronic van der Waals attraction between two molecules in contact is due practically entirely to interactions of pairs of atoms (in the two molecules) which are themselves in contact; and the magnitude of the attraction is determined by the number of pairs of atoms which can be brought into contact. In consequence, two molecules which can bring large portions of their surfaces into close-fitting juxtaposition will in general show much stronger mutual attraction than two molecules with less extensive complementariness of surface topography.

Other types of molecular interactions result from the possession of a permanent electric charge, electric dipole moment, or electric moment of higher order by one or both of the interacting molecules. The effects of these charges and moments have been classified in various ways, as ion-ion forces, dipole-dipole forces, forces of electronic polarization of one molecule in the dipole field of another, etc. All electrostatic interactions are very much smaller in water than in a medium of low dielectric constant, and it can be shown by calculation, making use of known values of the effective dielectric constant of water for charges a given distance apart (24), that in general these electric forces are of minor importance, except when an isolated or essentially isolated electric charge is involved. The electrostatic attraction of a positive group such as a substituted ammonium ion and a negative group such as a carboxyl ion becomes significantly strong, with bond energy 5 kilocalories per mole or more, if the structure of the molecules containing the groups is such that they can come into juxtaposition.

A type of intermolecular attractive force which ranks in importance with the electronic van der Waals attraction and the attraction of oppositely charged groups is that associated with the structural feature called the *hydrogen bond.* The importance and generality of occurrence of the hydrogen bond were first pointed out in 1920 by Latimer and Rodebush (25) and summaries of the properties of the bond are given in the monographs quoted above. A hydrogen bond results from the attraction of a hydrogen atom attached to one electronegative atom for an unshared electron pair of another electronegative atom. The strength of a hydrogen bond depends on the electronegativity of the two atoms which are bonded together by hydrogen; fluorine, oxygen, and nitrogen, the

most electronegative of all atoms, are the atoms which form the strongest hydrogen bonds. The energy of a hydrogen bond between two of these atoms is of the order of magnitude of 5 kcal. per mole. This is so large as to have a very important effect on the intermolecular interactions of molecules capable of forming hydrogen bonds and on the properties of the substances consisting of these molecules.

In synthesizing our knowledge of intermolecular forces and of immunological phenomena into a definite picture of the antigen-antibody bond the immunological property of greatest significance is the specificity of the combining power of antibody for the immunizing antigen.

The forces of van der Waals attraction, hydrogen-bond formation, and interaction of electrically charged groups are in themselves not specific; each atom of a molecule attracts every other atom of another molecule by van der Waals attraction, each hydrogen atom attached to an electronegative atom attracts every other electronegative atom with an unshared electron pair which comes near it, and each electrically charged group attracts every other oppositely charged group in its neighborhood. The van der Waals repulsive forces which determine the van der Waals radii of atoms also are not specific; each atom in a molecule repels every other atom of another molecule, holding it at a distance corresponding to the sum of the pertinent van der Waals radii. We see, however, that specificity can arise in the interaction of large molecules as a result of the shapes of the molecules. Two large molecules may have such spatial configurations that the surface of one cannot be brought into contact with the surface of the other except at a few isolated points. In such a case the total electronic van der Waals attraction between the two molecules would be small, because only the pairs of atoms near these few isolated points of contact would contribute appreciably to this interaction, and, moreover, the distribution of hydrogen-bond forming groups and of positively and negatively charged groups of two molecules might be such that only a small fraction of these groups could be brought into effective interaction with one another for any position and orientation of one molecule with respect to the other; the energy of attraction of these two molecules would then be small. If, on the other hand, the two molecules possessed such mutually complementary configurations that the surface of one conformed closely to the surface of the other, if, moreover, the electrically charged groups of one molecule and those of the other were so located that oppositely charged groups were brought close together as the molecules came into conformation with one another, and if the hydrogen-bond forming groups were also so placed as to form the maximum number of hydrogen bonds, the total energy of interaction would be very great, and the two molecules would attract one another very strongly. We see that this strong attraction might be highly specific in the case of large molecules which could bring large areas of their surfaces into close contact. A molecule would hence show strong attraction for another molecule which possessed complete complementariness in surface configuration and distribution of active electrically charged and hydrogen-bond forming groups, somewhat weaker attraction for those molecules

with approximate but not complete complementariness to it, and only very weak attraction for all other molecules.

This specificity through complementariness of structure of the two interacting molecules would be more or less complete, depending on the greater or smaller surface area of the two molecules involved in the interaction. It may be emphasized that this explanation of specificity as due to a complementariness in structure which permits non-specific intermolecular forces to come into fuller operation than would be possible for non-complementary structures is the only explanation which the present knowledge of molecular structure and intermolecular forces provides.

This theory of structural complementariness of antigen and antibody was first suggested, in less detailed form than above, by Breinl and Haurowitz (11), Alexander (12), and Mudd (13). A detailed discussion of the structure of antibodies and of a postulated method of their formation has been presented by Pauling (17), who has also reviewed the evidence supporting the theory of complementariness.

It was suggested by Breinl and Haurowitz and by Mudd that the effect of an antigen in determining the structure of an antibody might involve the ordering of the amino-acid residues in the polypeptide chains in a way different from that in the normal globulin. Rothen and Landsteiner (26) then pointed out that the possibility of different ways of folding the same polypeptide chain is worth considering, and this postulate was amplified by Pauling (17), who assumed that all antibody molecules contain the same polypeptide chains as normal globulin, and differ from normal globulin only in the configuration of the chains. This assumption was made because it permits the formulation of a simple proposed mechanism of manufacture of specific antibodies. An antibody molecule, capable of existing in any one of a great number of configurations with nearly the same energy, is synthesized, except for the final folding step, in the same way as normal globulin. If no foreign substance is present, the chain then folds into a stable configuration, characteristic of normal globulin; but if an antigen molecule is present, the chain folds into a configuration stable in the presence of the antigen, that is, into a configuration complementary to that of a portion of the surface of the antigen molecule. This explanation of the ability of an animal to form antibodies with considerable specificity for an apparently unlimited number of different antigens (27), as shown especially by the work of Landsteiner (10), is compatible with the principles of structural chemistry and thermodynamics as well as with the immunological evidence.

To illustrate the way in which the complementariness theory accounts for many reported observations we shall mention only one point, taken from the great body of results on azoproteins obtained by Landsteiner. He observed a pronounced cross reaction between an azoprotein made from *m*-aminobenzoic acid and an antiserum to an azoprotein made from 4-chloro-3-aminobenzoic acid and a different protein, but no reaction with the haptenic groups reversed. The explanation of this is that the antibody to the 3-azo-4-chlorobenzoic acid group conforms closely to this haptenic group, allowing either this group or the

3-azobenzoic acid group, which differs in the replacement of the chlorine atom by a smaller atom, hydrogen, to fit into the complementary cavity in the antibody; but the 3-azo-4-chlorobenzoic acid group cannot fit into a cavity designed for the smaller haptenic group, and so the reverse cross reaction does not occur. In a quantitative extension of Landsteiner's work on hapten inhibition of precipitation reactions of simple polyhaptenic substances (10), Pauling and collaborators (28) have recently reported a great deal of evidence in support of the complementariness theory. They interpreted their results on the inhibition of the precipitation reaction between dyes containing *p*-azophenylarsonic acid groups and antisera to hapten-homologous azoproteins to obtain numerical values of the strength of the bonds formed by these antibodies with over twenty-five different haptens. The observed correlation between the bond strengths and the structure of the haptens is that which would be expected from the complementariness theory.

This theory is not greatly different from some earlier proposals, such as Ehrlich's lock-and-key analogy (29), but it differs greatly from others. For example, Buchner (30) considered that antigen molecules are split up and incorporated into the antibody molecules, thus imparting specificity to them. This theory or a closely related theory has been supported by many people, including Burnet (31), who proposed a mechanism for the manufacture of antibodies in the image of the antigens: the antigens act as templates for the manufacture by the body of specific enzymes, which then serve as the molds for the production of antibodies similar to the original antigens. Until recently there has been no suggestion as to why antibodies similar in structure to an antigen should combine specifically with it. Recently, however, Jordan (32) has stated that a strong attraction would occur between such identical or nearly identical molecules because of the quantum-mechanical resonance phenomenon; this has been denied by Pauling and Delbrück (33), who pointed out that the resonance energy would be so small as to be ineffective. Chemical evidence against the identity of antibodies and specific antigens has been presented by many authors, of whom the most recent are Haurowitz, Vardar, and Schwerin (34).

Forty years ago there was under way a keen controversy between Ehrlich and Bordet, and their respective supporters, as to whether the bonds between antibodies and antigens are chemical bonds or are physical forces of the sort producing surface phenomena such as adsorption. The modern point of view resolves this argument, but not in favor of either side; in fact, as in recent years an understanding has been obtained of the forces responsible for surface phenomena it has been found that these forces are the same as those which are operative in chemical reactions, so that the old distinction between chemical and physical forces has lost most of its meaning.

THE NATURE OF THE PRECIPITATE. Under suitable conditions (salt concentration, antibody-antigen ratio, etc.) the first stage of combination of antibody and antigen, which may make itself evident in change in toxicity or other properties of the antigen, is followed by precipitation. There has been much discussion as to whether or not this second stage is specific, like the first stage,

or is non-specific. Direct experimental evidence on this point, while not conclusive, favors the view that the reaction is specific. The most pertinent observations are those on the agglutination of mixed cellular antigens by mixed antisera; Topley and collaborators (35) noted the formation of separate clumps by the different cells, whereas Abramson (36) observed mixed clumping. Hooker and Boyd (37) found that mixed human and chicken erythrocytes gave separate clumps under some conditions and mixed clumps under other conditions. The fact that separate clumping is observed at all strongly favors the concept of a specific second stage, since mixed clumping might result from mechanical intertwining of specific clumps, whereas separate clumping would hardly be expected to result from non-specific interaction. Heidelberger has pointed out that in those cases where mixed clumping takes place the cells used were either very large or of greatly different sizes (38).

A reasonable theory of agglutination and precipitation, the framework theory (lattice theory[1]), was proposed in 1934 by Marrack (6), and has received strong support from the theoretical considerations and experiments of Heidelberger (9, 14, 15) and Pauling (18, 19, 20, 28) and their collaborators.

It is clear that, after we have accepted a mechanism for the specific attachment of antibody molecules to a cellular antigen, the simplest possible explanation of the agglutination of the cells is that it results from the same mechanism; if an antibody molecule had the power of specific attachment to two cells, it could form specific bonds with the two cells and thus hold them together, and the repetition of this process would lead to the formation of larger and larger clumps. Specific precipitation of antibodies and molecular antigens would result from the same mechanism if both antibody molecules and antigen molecules were multivalent (capable of forming two or more antigen-antibody bonds); larger and larger complexes A—B, A—B—A, A—B—A—B, etc., would form until the aggregates became macroscopic in size. The evidence supporting the framework theory has been reviewed by Marrack (6), Heidelberger (14), and Pauling (17); some of it, including that provided by recent work, is presented in the following section in connection with a discussion of the valence of antibody molecules.

The first of the theories of non-specific precipitation is the theory of neutralization of electrical charges. This theory was supported by many early investigators, who were attracted by the analogy with the well-known phenomenon of the mutual precipitation of oppositely charged colloids. Teague and Field (39) investigated the charges of agglutinins and bacteria and concluded that the former are positively and the latter negatively charged; it is now known, however, as the result of the application of improved experimental methods, that under ordinary conditions (normal hydrogen-ion and salt concentrations) antibody molecules and most antigens are negatively charged, and the theory of neutralization has in consequence been abandoned.

(The failure of precipitation or agglutination to occur in antigen-antibody

[1] We have adopted the name "framework theory" instead of "lattice theory" because of our belief that the framework of antibody-antigen precipitates does not usually have the regularity of structure which would be indicated by use of the latter expression.

systems with low salt concentration is, indeed, attributed to the electrostatic repulsion of the negatively-charged complexes in solution, which prevents the formation of large aggregates; agglutination or precipitation may occur in the presence of salt, the cations of which neutralize the negative charges of the complexes.)

Another theory of non-specific precipitation which was proposed soon after the discovery of the precipitation reaction is that the reaction results from the formation of a hydrophobic colloid, which precipitates in the presence of electrolytes. This theory has been revived recently by Eagle (40) and by Hooker and Boyd (37). Eagle's suggestion that the polar groups of the antigen which are assumed to be responsible for its solubility are masked by a layer of antibody molecules, which themselves turn their polar groups inward and present only non-polar groups toward the solvent, has been discussed by Marrack (6), who has marshalled some arguments against it. An important argument is that particles can be agglutinated by an amount of antibody very much smaller than the amount required to coat their surface; the most recently reported experiments of this sort (41) indicate that azoerythrocytes can be agglutinated by less than 0.02 per cent as much antibody as would cover their surface with a layer 3.5 Å thick.

Hooker and Boyd (37) have presented several arguments in support of the thesis that "... particles grow to visible size by the indiscriminate and non-specific accretion of other related or unrelated, small or large, aggregates whose primary nuclei are molecules or particles of antigen coated with antibody-globulin." As additional evidence for this concept and against the framework theory Boyd and Hooker (42) reported their failure to inhibit the agglutination of erythrocytes by use of an excess of hemagglutinin. In our opinion the fact that inhibition of agglutination of particles (43) as well as of precipitation of molecular antigens (44) by excess antibody has been observed gives strong support to the framework theory. The failure of inhibition to occur under ordinary circumstances may be due to the difficulty in saturating the multivalent antigens, especially cellular antigens with thousands of combining groups, as is indicated by the theories of Hershey (45) and Pauling (17). In particular, the experiments of Heidelberger and Kabat (43) on the agglutination by untreated pneumococci of pneumococci coated with antibody and then thoroughly washed are most easily explained by the framework theory. Hooker and Boyd (46, 47) have recently proposed the theory that precipitation of antibody by polyhaptenic dyes may result from the action of the dye molecules in pulling the antibody molecules to which they are bonded so tightly together as to prevent the solvent from reaching the polar groups. This theory seems to be incompatible with our observation (18) that in general the dyes of smaller molecular size, which according to Boyd's theory should pull the molecules more closely together, in fact precipitate less completely than those of larger molecular size.

COMPOSITION OF ANTIBODY-ANTIGEN PRECIPITATES AND VALENCE OF ANTIBODY. An essential requirement for agglutination or precipitation according to the framework theory is that both antigen and antibody be multivalent. The

experimental observations which indicate multivalence of antigens and of agglutinins and precipitins have been summarized by Marrack (6), Heidelberger (14), and Pauling (17).

The most straightforward evidence for the necessary multivalence of antigen is given by experiments on the reactions of antibodies with simple substances of known structure. Subsequent to Landsteiner's discovery (10) that simple haptens inhibit the precipitation and agglutination reactions by forming soluble complexes with antibody, it was found by Landsteiner and Van der Scheer (21) that simple substances containing two or more haptenic groups form precipitates with hapten-homologous antibodies. We have shown that of the twenty-seven simple substances containing phenylarsonic acid groups which were tested with antisera made by injecting rabbits with azoprotein made from *p*-arsanilic acid each of those (twenty in number) which contained two or more of the haptenic groups gave the precipitation reaction, whereas none of the monohaptenic substances formed precipitates. These facts support the framework theory strongly.

(The failure to obtain precipitates with some polyhaptenic substances reported by Hooker and Boyd (46) and Boyd (47) may have been due to their failure to work under conditions favorable to precipitation. We have obtained precipitates with some of the same substances, and have observed that substances which give precipitates with strong antisera may fail to do so with weak antisera.)

Experiments which have been reported on the number of haptenic groups per azoprotein molecule necessary for precipitation with hapten-homologous antibody (48, 49) and some of our unpublished results indicate that a few groups are needed, but so far they have not been precise enough to distinguish between 1 and 2 as the minimum.

Direct proof of the bivalence of diphtheria antitoxin is given by the studies of the antitoxin in presence of an excess of toxin with use of the ultracentrifuge, which showed that the complexes ToxAntitox and Tox_2Antitox exist in the solution (50).

The fact that slides can be coated with alternate unimolecular layers of antigen and antibody in specific combination (51, 52) indicates effective bivalence of antibody molecules as well as antigen molecules.

It has long been known that in antigen-antibody precipitates molecules of antibody are present in larger numbers than those of antigen, the antibody-antigen molecular ratio being considerably greater than unity for nearly all systems (6, p. 161; 53). This was shown convincingly by the accurate quantitative investigations of Heidelberger and his collaborators (54). A simple explanation of this fact, which does not follow directly from the framework theory in its original form (6, 14), is given by the theory as modified by Pauling (17), who made the assumption that antibodies in general are at the most bivalent. (This assumption was made because his proposed structural theory of the process of formation of antibodies is such as to make unlikely the occurrence of antibodies of higher valence.) The maximum valence N of antigens toward homologous antibodies is assumed to be determined by the sizes and shapes of the

antigen and antibody molecules, being equal to the number of antibody molecules which, when bonded to the antigen molecule, can be packed around it. If the antigen and antibody molecules were spheres of the same size, this number would be $N = 12$; for smaller antigen molecules it would be smaller, and for larger ones it would be larger.

The predicted antibody-antigen molecular ratio for small antigen molecules would be $N/2$ at the equivalence zone, with the maximum valences of both antibody and antigen effective; the limiting values of the ratio for antigen excess would be 1, and for antibody excess $N - 1$. For very large antigens the expected ratio at the equivalence zone would be less than $N/2$. These predictions are in reasonably good agreement with Heidelberger's observations (54, 17), which correspond to the following values of N: ovalbumin and R-salt-azobiphenylazoovalbumin (molecular weight 40,000–46,000), $N = 6$; serum albumin (m.w. 67,000), $N = 6$ to 8; thyroglobulin (m.w. 700,000), $N = 30$ to 40. Data of other investigators (55, 56, 57, 58, 59, 60, 61) correspond to similar values of N, with assumed bivalence of antigen.

If the valence of the antigen were known, measurement of the antibody-antigen molecular ratio could be interpreted to give the valence of antibody molecules. The only reliable experiments of this sort which have been reported so far are those of Pauling, Pressman, and Ikeda (20) with simple antigens of known structure. They found that the dihaptenic antigens 2-methyl-4,6-di(*p*-azophenylarsonic acid)phenol and 2-methyl-4,6-di(*p*-azobenzene(p-azophenylarsonic acid))phenol gave with antisera homologous to the *p*-azophenylarsonic acid group precipitates with the same molecular ratio throughout the range of relative concentrations from antibody excess to antigen excess. This is what would be expected if both antibody and dihaptenic antigen were bivalent, the predicted molecular ratio under all conditions then being 1, corresponding to the structure —A—B—A—B—A—B—A—B— for the precipitate. The observed molecular ratio (average of 119 analyses) was 0.75. The same independence of molecular ratio on relative concentration was found also for the four trihaptenic and tetrahaptenic substances studied; this was interpreted as resulting from the effective bivalence of these molecules also, as the result of their small size in comparison with the antibody molecules. The average molecular ratios found for the trihaptenic and tetrahaptenic substances, 0.85 and 0.83, respectively, are only slightly less than unity. These results, with assumed effective bivalence of the antigens, indicate for antibody molecules the effective valence 2.3.

Substantiating evidence for the multivalence of precipitating antibodies is provided by the observations which have been interpreted as resulting from the presence in antisera of antibodies with a valence of one. Heidelberger and Kendall (62) demonstrated the presence in rabbit antisera of univalent antibodies, which are able to combine specifically with antigen but are not able, in the absence of multivalent antibodies, to form precipitates. These univalent antibodies also occur in considerable amount in horse antisera; they seem to be produced in large amount by the first injections of ovalbumin into horses, precipitating (multivalent) antibody being formed only on repeated

injection (63, 64). It is probable that univalent antibody confers on horse antitoxins the peculiar properties which they show, in particular the pronounced prezone (region of antitoxin excess in which precipitation does not occur). The change in properties of antisera on heat treatment (65, 66, 67) or treatment with formaldehyde (9) or other denaturing agent is probably due to the conversion of bivalent antibody to univalent antibody by destruction of one of the combining regions.

QUANTITATIVE THEORIES OF THE PRECIPITATION REACTION. Although the quantitative physico-chemical treatment of immunological phenomena was begun early in the present century, by Arrhenius and Madsen (1, 68), it is only during the last decade that significant progress has been made. The reason for the delay is not far to seek—it lies in the fact that the mathematical treatment of numerical data of low accuracy has little significance so long as a sound qualitative understanding of the phenomenon has not been developed. We may mention in illustration Arrhenius' discussion (1, p. 147) of the formula $C = K B^{2/3}$ which he found to express the relation between the amount C of agglutinin bound by bacterial cells and the amount B of free agglutinin; Arrhenius interpreted this equation (whose validity for the complex system we would ascribe to the accidental distribution of the heterogeneous antiserum) as showing that the agglutinin molecules are divided between two solvents, one within and one without the bacterial cells, and that three molecules of the bound agglutinin are formed from two of the free substance.

Recent theories are of two kinds: those based on thermodynamic equilibrium among the reacting substances, and those based on the rates of reactions under non-equilibrium conditions. There has been considerable discussion as to whether or not immunological reactions are reversible—whether, for example, an antigen-antibody precipitate is soluble, and is in equilibrium with free antigen and antibody in solution. We know from general principles, however, that, given time enough, every system reaches equilibrium, and every material is more or less soluble; the questions of interest deal rather with such quantitative points as the length of time required for the system to reach equilibrium, and the magnitude of the solute concentrations in equilibrium with the precipitate.

That the precipitation reaction in some cases reaches equilibrium in the hours or days usually allowed it is shown by various experiments on solution of the precipitate by salt (69), acid (70), alkali (71), and excess antigen, even after ageing for several months (72), including experiments in which there was variation in the method of approaching equilibrium (19).

Experiments on the Danysz phenomenon (73) and other related experiments indicate that a long time—many days—is needed for equilibrium to be approached for reactions involving change in composition of antigen-antibody precipitates.

The first quantitative theory which we shall discuss, that of Heidelberger and Kendall (14), was based on consideration of the rate of antigen-antibody combination under non-equilibrium conditions. The authors assumed that antigen A and antibody B first react completely and rapidly to form the com-

plex AB, which uses up all the A (B being assumed present in excess). There then occur two competing slow reactions:

$$\begin{aligned} B + AB &\rightarrow AB_2 \\ AB + AB &\rightarrow A_2B_2 \end{aligned}$$

The rates of formation of AB_2 (total number of units α) and A_2B_2 (total number of units β) are

$$\frac{d\alpha}{dt} = K[B][AB]$$

and

$$\frac{d\beta}{dt} = K'[AB]^2$$

in accordance with the laws of chemical kinetics. It is assumed arbitrarily that $K = K'$ and that the reactions are not reversible; by integration over the course of the reaction until the solution is exhausted of AB there is obtained to represent the composition of the precipitate, which consists of all the AB_2 and A_2B_2 formed, the equation

$$\frac{y}{a} = 2R - \frac{R^2 a}{b_0} \qquad (1)$$

in which

y = milligrams of antibody precipitated
a = milligrams of antigen added
b_0 = total milligrams of antibody
R = antibody/antigen weight ratio at equivalence point

This equation for the composition of the precipitate, which corresponds to change from $2R$ at large antibody excess to R at the equivalence point, has been shown (15) to be in satisfactory agreement with the excellent experimental data obtained by the authors. In view of the arbitrary and unlikely assumptions originally used for its derivation, it is gratifying that Kendall himself (74) has recently derived the equation in another way, and that we have found (unpublished work) that the equation is obtained as an approximation from general considerations of chemical equilibrium when the assumption is made that the ratio may vary between the limits $2R$ and R and an expansion is made in powers of a/b.

Kendall's derivation is essentially the following. (He considers also some more general cases.)

Let antibody and antigen both be bivalent. For B_0 and A_0 molecules of antibody and antigen, respectively, there are $2B_0$ and $2A_0$ combining groups. Assume, for antibody excess, that all of the $2A_0$ antigen groups are bonded to antibody groups, and that they are distributed at random among the $2B_0$ antibody groups, without regard to whether or not the antibody molecule is already bonded at the other end. Since the chance that an antibody group is

bound is $2A_0/2B_0$, the chance that it is free is $1—A_0/B_0$, and the fraction of antibody molecules free at both ends is $(1—A_0/B_0)^2$. The fraction not free at both ends is $1—(1—A_0/B_0)^2 = 2A_0/B_0—(A_0/B_0)^2$, and the number not free at both ends is this multiplied by the total number, B_0. If it be assumed that all antibody molecules not free at both ends are carried down in the precipitate with the antigen molecules the molecular ratio for the precipitate becomes

$$\frac{B_{pp}}{A_{pp}} = 2 - A_0/B_0 \tag{2}$$

and, introducing the ratio R of molecular weights, the weight ratio is found to be

$$\frac{y}{a} = 2R - R^2\frac{a}{b_0} \tag{3}$$

This is identical with equation 1.

Similar considerations have also been used by Ghosh (75) for the derivation of related equations.

An involved theory of antigen-antibody equilibria based in part on probability considerations has been extensively developed by Hershey (45). The theory, in common with others based on multivalent antigen and antibody, is in qualitative and rough quantitative accord with experiment.

The only theory of the precipitation reaction which, following the program begun by Arrhenius, has been developed by straightforward application of the principles of chemical equilibrium is that of Pauling, Pressman, Campbell and Ikeda (19). This theory applies only to relatively simple systems, namely, those composed of bivalent antigen and bivalent antibody, univalent hapten, certain soluble complexes, and precipitate with invariant composition AB.

In order to show the nature of the treatment, we present here the derivation of the equation for the amount of precipitate formed in absence of hapten, generalized over the earlier treatment by consideration also of the complex AB_2.

Let the molecular species A, B, AB, A_2B, and AB_2 in solution be in equilibrium with each other and with solid AB_{pp}. We represent the concentrations of the five solutes by the symbols

$$\begin{aligned} [A] &= \alpha \\ [B] &= \beta \\ [AB] &= s \\ [A_2B] &= a \\ [AB_2] &= b \end{aligned}$$

The quantities α, β, a, and b are variable, whereas s is constant for a given system with precipitate present; it is the solubility of the precipitate. The equilibrium expressions for the three reactions

$$\begin{aligned} A + B &= AB \\ A + AB &= A_2B \\ B + AB &= AB_2 \end{aligned}$$

are respectively

$$\frac{s}{\alpha\beta} = 4K \tag{4}$$

$$\frac{a}{\alpha s} = K \tag{5}$$

$$\frac{b}{\beta s} = K'' \tag{6}$$

For simplicity we have assumed that each of the bonds in the complex A—B—A has the same strength as the bond in AB; this is probably a good approximation, in view of the fact that the two bonding regions are probably far apart on the large antibody molecules. (The theory can be carried through without this assumption.) The constant K corresponds to equilibrium for one antibody valence and one antigen valence, and the factor 4 is an entropy factor or symmetry factor. We use K'' rather than K for the second bond in AB_2 because steric repulsion between the two antibody molecules attached to the same small antigen would be expected to decrease the stability of this complex.

The expressions for the total amounts of antigen and antibody in the system (per unit volume of solution) are

$$AB_{pp} + s + \alpha + 2a + b = A_{total} \tag{7}$$

and

$$AB_{pp} + s + \beta + a + 2b = B_{total} \tag{8}$$

Subtracting equation 8 from 7 we obtain

$$\alpha - \beta + a - b = A_{total} - B_{total}$$

Eliminating β, a, and b with the use of equations 4, 5, and 6 we obtain

$$\alpha - \frac{s}{4K\alpha} + Ks\alpha - \frac{K''s^2}{4K\alpha} = A_{total} - B_{total}$$

This quadratic equation in α gives on solution

$$\alpha = \frac{1}{2(1+Ks)}\{(A_{total} - B_{total} + [s(1+K''s)(1+Ks)/K + (A_{total} - B_{total})^2]^{\frac{1}{2}} \tag{9}$$

(The positive rather than the negative sign before the radical is seen to be correct by the consideration of limiting cases.) From equation 7 we find on eliminating a and b the expression

$$AB_{pp} = A_{total} - s - (1+Ks)\alpha - \frac{K''s^2}{4K\alpha} \tag{10}$$

Equations 9 and 10 give the solution to our problem; from 9 the value of α is to be found in terms of A_{total} and B_{total} and the parameters of the system s, K, and K'', and this on substitution in 10 gives the amount of precipitate.

It is shown in the original paper how this equation (with $K'' = 0$) accounts for many observed properties of antigen-antibody systems.

Future progress which may be anticipated involves the extension of this straightforward thermodynamic treatment to include the case of variable composition and randomness of structure of the solid phase. This will require the development of satisfactory approximate expressions for the free energy of such a solid phase, by application of the methods of statistical mechanics on the basis of sound structural concepts. This problem is not an easy one; but fortunately promising methods for attacking it have been developed in recent years by able theoretical physicists interested in the problem of the stability of alloys with greater or smaller degree of randomness of atomic arrangement, and we may be confident that great progress will soon be made in the formulation of a satisfactory quantitative theory of the precipitation reaction.

SUMMARY

The forces responsible for combination and attraction of antigen and antibody molecules may be classified as electronic van der Waals attraction, Coulomb attraction, attraction of electric dipoles or multipoles, formation of hydrogen bonds, etc. The specificity of interaction of antigen and antibody molecules arises from their structural complementariness, which permits close contact of the molecules over sufficient area for these weak forces to co-operate in forming a strong antigen-antibody bond.

The weight of evidence indicates that further combination of the initial antigen-antibody complexes to form a precipitate is a specific rather than a nonspecific reaction and is due to a continuation of the primary combination step to form a framework structure of alternate antigen and antibody molecules.

Furthermore it appears that both precipitating antigen and precipitating antibody must be multivalent, at least bivalent.

The more recent quantitative theories of the precipitation reaction are discussed.

REFERENCES

(1) ARRHENIUS, S. Immunochemistry. Macmillan and Co., 1907, p. 31.

(2) PAULING, L. The nature of the chemical bond and the structure of molecules and crystals. Cornell University Press, Ithaca, N. Y., 2nd ed., 1940.

(3) KRAUS, R. Wien. klin. Wchnschr. **10:** 736, 1897.

(4) KRAUS, R. In KOLLE and WASSERMANN. Handbuch path. Microörganismen **4:** 592, 1904.

(5) DEAN, H. R. A system of bacteriology, (V. 6—Immunity p. 424). Medical Research Council, His Majesty's Stationery Office, London, 1931.

(6) MARRACK, J. R. The chemistry of antigens and antibodies. Report no. 230 of the Medical Research Council, His Majesty's Stationery Office, London, 1934; second edition, 1938.

(7) HARTLEY, P. Brit. J. Exper. Path. **7:** 55, 1926.

(8) FELTON, L. D. AND G. H. BAILEY. J. Infect. Dis. **38:** 131, 1926.
(9) HEIDELBERGER, M. ET AL. J. Exper. Med. **64:** 161, 1936; **67:** 181, 1938; **68:** 913, 1938.
(10) LANDSTEINER, K. The specificity of serological reactions. C. C. Thomas, Springfield, Ill., 1936.
(11) BREINL, F. AND F. HAUROWITZ. Ztschr. physiol. Chem. **192:** 45, 1930.
(12) ALEXANDER, J. Protoplasma **14:** 296, 1931.
(13) MUDD, S. J. Immunol. **23:** 423, 1932.
(14) HEIDELBERGER, M. Chem. Rev. **24:** 323, 1939; J. Exper. Med. **61:** 563, 1935.
(15) HEIDELBERGER, M. AND F. E. KENDALL. J. Exper. Med. **50:** 809, 1929.
(16) HAUROWITZ, F. AND F. BREINL. Ztschr. physiol. Chem. **214:** 111, 1933.
(17) PAULING, L. J. Am. Chem. Soc. **62:** 2643, 1940.
(18) PAULING, L., D. H. CAMPBELL AND D. PRESSMAN. Proc. Nat. Acad. Sci. **27:** 125, 1941.
PAULING, L., D. PRESSMAN, D. H. CAMPBELL, C. IKEDA AND M. IKAWA. J. Am. Chem. Soc. **64:** 2994, 1942.
(19) PAULING, L., D. PRESSMAN, D. H. CAMPBELL AND C. IKEDA. J. Am. Chem. Soc. **64:** 3003, 1942.
(20) PAULING, L., D. PRESSMAN AND C. IKEDA. J. Am. Chem. Soc. **64:** 3010, 1942.
(21) LANDSTEINER, K. AND J. VAN DER SCHEER. Proc. Soc. Exper. Biol. and Med. **29:** 747, 1932; J. Exper. Med., **56:** 399, 1932.
(22) RICE, O. K. Electronic structure and chemical binding. McGraw-Hill Book Co., New York, 1940.
(23) LONDON, F. Ztschr. Physik **63:** 245, 1930.
(24) SCHWARZENBACH, G. Ztschr. physik. Chem. **A176:** 133, 1936.
(25) LATIMER, W. M. AND W. H. RODEBUSH. J. Am. Chem. Soc. **42:** 1419, 1920.
(26) ROTHEN, A. AND K. LANDSTEINER. Science **90:** 65, 1939.
(27) HEKTOEN, L. AND A. K. BOOR. J. Infect. Dis. **48:** 588, 1931.
(28) PRESSMAN, D., D. H. BROWN AND L. PAULING. J. Am. Chem. Soc. **62:** 3015, 1942.
(29) EHRLICH, P. Studies on immunity. John Wiley and Sons, New York, 1906.
(30) BUCHNER, H. Münch. med. Wchnschr. **40:** 449, 1893; **47:** 277, 1900.
(31) BURNET, F. M. The production of antibodies. Macmillan & Co., Ltd., Melbourne, Australia, 1941, Chap. X.
(32) JORDAN, P. Ztschr. Physik **113:** 431, 1939; Ztschr. f. Immunitätsforsch. **97:** 330, 1940; Naturwissenschaften **29:** 89, 1941.
(33) PAULING, L. AND M. DELBRÜCK. Science **92:** 77, 1940.
(34) HAUROWITZ, F., M. VARDAR AND P. SCHWERIN. J. Immunol. **43:** 327, 1942.
(35) TOPLEY, W. C. C., J. WILSON AND J. T. DUNCAN. Brit. J. Exper. Path. **16:** 116, 1935.
(36) ABRAMSON, H. A. Nature **135:** 995, 1935.
(37) HOOKER, S. B. AND W. C. BOYD. J. Immunol. **33:** 337, 1937.
(38) HEIDELBERGER, M. Bact. Rev. **3:** 49, 1939.
(39) TEAGUE, O. AND C. W. FIELD. J. Exper. Med. **9:** 86, 1907.
(40) EAGLE, H. J. Immunol. **18:** 393, 1930.
(41) PRESSMAN, D., D. H. CAMPBELL AND L. PAULING. J. Immunol. **44:** 101, 1942.
(42) BOYD, W. C. AND S. B. HOOKER. Proc. Soc. Exper. Biol. and Med. **39:** 491, 1938.
(43) HEIDELBERGER, M. AND E. A. KABAT. J. Exper. Med. **65:** 885, 1937.
(44) DEAN, H. R. Proc. Roy. Soc. Med. **5:** 62, 1911.
(45) HERSHEY, A. D. J. Immunol. **42:** 455, 1941.
(46) HOOKER, S. B. AND W. C. BOYD. J. Immunol. **42:** 419, 1941.
(47) BOYD, W. C. J. Exper. Med. **75:** 407, 1942.
(48) HOOKER, S. B. AND W. C. BOYD. J. Immunol. **23:** 446, 1932.
(49) HAUROWITZ, F., F. KRAUS AND F. MARX. Ztschr. physiol. Chem. **245:** 23, 1936.
(50) PAPPENHEIMER, A. M., JR., H. P. LUNDGREN AND J. W. WILLIAMS. J. Exper. Med. **71:** 247, 1940.
(51) PORTER, E. F. AND A. M. PAPPENHEIMER, JR. J. Exper. Med. **69:** 755, 1939.

(52) FOURT, W., W. D. HARKINS AND G. G. WRIGHT. Abstract 98th Meeting Am. Chem. Soc., 1939.
(53) MARRACK, J. R. Ann. Rev. Biochem. **11:** 629, 1942.
(54) HEIDELBERGER, M. J. Am. Chem. Soc. **60:** 242, 1938.
(55) BOYD, W. C. AND S. B. HOOKER. J. Gen. Physiol. **17:** 341, 1934; **22:** 281, 1939.
(56) KLECZKOWSKI, A. Brit. J. Exper. Path. **22:** 44, 1941.
(57) LIU, S. C. AND H. WU. Chinese J. Physiol. **15:** 237, 1940.
(58) WU, H., L. H. CHENG AND C. P. LI. Proc. Soc. Exper. Biol. **25:** 407, 1928.
(59) CULBERTSON, J. T. J. Immunol. **23:** 439, 1932.
(60) MARRACK, J. R. AND F. C. SMITH. Brit. J. Exper. Med. **12:** 30, 1931.
(61) PAPPENHEIMER, A. M., JR. AND E. S. ROBINSON. J. Immunol. **32:** 291, 1937.
(62) HEIDELBERGER, M. AND F. E. KENDALL. J. Exper. Med. **62:** 697, 1935.
(63) PAPPENHEIMER, A. M., JR. J. Exper. Med. **71:** 263, 1940.
(64) HEIDELBERGER, M., H. P. TREFFERS AND M. MAYER. J. Exper. Med. **71:** 271, 1940.
(65) HOOKER, S. B. AND W. C. BOYD. Ann. N. Y. Acad. Sci. **43:** 107, 1942.
(66) SHIBLEY, C. S. J. Exper. Med. **50:** 825, 1929.
(67) TYLER, A. Biol. Bull. **81:** 190, 1941.
(68) MADSEN, T. Comm. de l'institut serotherapique de l'Etat Danois, 1907–13.
(69) HEIDELBERGER, M. AND F. E. KENDALL. J. Exper. Med. **64:** 161, 1936.
(70) LIU, S. AND H. WU. Chinese J. Physiol. **15:** 465, 1940.
(71) FELTON, L. D. J. Immunol. **22:** 453, 1932.
(72) BOYD, W. C. J. Immunol. **38:** 143, 1940.
(73) HEALEY, M. AND S. PINFIELD. Brit. J. Exper. Path. **16:** 535, 1935.
(74) KENDALL, F. E. Ann. N. Y. Acad. Sci. **43:** 85, 1942.
(75) GHOSH, B. N. Ind. J. Med. Research **23:** 285, 1935.